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Growth factors, Cytokines and VEGF in human neoplastic and inflammatory pathologies

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Chapter 3

Neurotrophins, their receptors and MIB-1 in Human GH-secreting Pituitary Adenomas : an immunohistochemical analysis

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Key words: Pituitary Gland – Adenoma – Neurotrophins – MIB-1 – Immunohistochemistry

Abstract

Pituitary Adenomas are a diverse group of tumors arising from the pituitary gland. Typically, they are small, slow – growing, hormonally inactive lesions that come to light as incidental findings on radiologic or postmortem examinations, although some small, slow – growing lesions with excessive hormonal activity may manifest with a clinical syndrome. The family of neurotrophins plays a key role in the development and maintenance of the pituitary endocrine cell function and in the regulation of hypothalamo – pituitary – adrenocortical axis activity. The objective of our experimental study was to investigate the localization of the neurotrophins, their relative receptors and detect the expression level of MIB-1 (anti-ki67) to determine if all these factors participate in the transformation and development of human pituitary adenomas. A very strong expression of NT-3 and its receptor TrKC was observed in the extracellular matrix (ECM) and vessel endothelium, together with a clear/marked presence of BDNF and its receptor TrKB, thus confirming their direct involvement in the progression of pituitary adenomas. On the contrary, NGF and its receptor TrKA and p75NTR were weakly expressed in the epithelial gland cells and the ECM.

Introduction

Pituitary Adenomas are a diverse group of tumors arising from the pituitary gland (1 – 2) which can be sub-classified as either functional or nonfunctional, depending on their hormonal activity in vivo (2 – 4). Small, slow – growing, hormonally inactive lesions are typically identified as incidental findings on radiologic or postmortem examinations: other small, slow – growing lesions with excessive hormonal activity can manifest with a clinical syndrome (2 – 4). Tumors that grow more rapidly, even if hormonally inactive, are capable of producing symptoms related to the presence of an intracranial mass, such as visual field disturbances. Given the small size of many pituitary tumors and their propensity to exist with only insidious, nonspecific symptoms, an accurate estimate of the incidence of pituitary adenomas in the general population appears challenging (4). Ezzat S et al found prolactinomas to be the most common form of pituitary adenoma (1). Burrow and co – workers (5) evaluated postmortem radiographic, macroscopic and microscopic findings in the pituitary tumors and found a significant incidence (11%) of prolactinoma in both men and women at all ages, despite the fact that the etiologies of most pituitary tumors remain unknown, as confirmed by more recent experimental findings (6 – 7). The family of neurotrophins plays a key role in the development and maintenance of nerve cell populations in the peripheral and central nervous system (8 – 12). Several lines of evidence suggest that neurotrophins, more particularly NGF, may be involved in pituitary endocrine cell function (13 – 14). Animal studies have shown that neurotrophins, especially NGF, play a dual role in pituitary physiology. Firstly, a local role, in the anterior pituitary gland, as a stimulator of differentiation and proliferation of somatomammotroph cells into mammotroph or lactotrope cells during development (15 – 18) as well as a stimulator of proliferation of the thyrotrope cells (18). Secondly, a systemic role as a neurohormone which is co – secreted with prolactin into the bloodstream (16). Furthermore, NGF may regulate endocrine function by stimulating the hypothalamo – pituitary – adrenocortical axis activity during stress responses, pregnancy and lactation (19). In vitro

studies revealed that escape from NGF control appears to be one of the mechanisms involved in the development and progression of human prolactinomas (20). NGF and Trk receptors have been identified in endocrine cells (cells containing conventional anterior – pituitary hormones) in the anterior pituitary gland of adult rats (14, 17 – 18), and Aguado et al (14) reported the presence of numerous endocrine cells containing TrkA and TrkB receptors in human anterior pituitary gland and some pituitary adenomas, suggesting a direct role of the neurotrophins in the normal development of the pituitary gland and in the progression of some pituitary adenomas. Previous observations found that prolactinomas, the most common pituitary tumor, express NGF and its receptors (TrkA and TrkB) and are therefore highly sensitive to the action of that neurotrophic factor. In fact, NGF mediates a long – lasting conversion of the more transformed dopamine – resistant prolactinomas into a differentiated, less malignant lactotroph – like phenotype re – expressing the D-2 receptor protein: the latter is very important in the pharmacological therapy of these tumors because D-2 receptor agonists are strongly effective in lowering plasma PRL levels in patients with prolactinomas (21). It has been also suggested that BDNF, expressed together with its receptor TrkB in the anterior lobe of the pituitary gland, is involved in the control of thyroid function, in the proliferation and differentiation of melanotrope cells and in the regulation of the hypothalamic – pituitary – adrenal axis (22 – 23). All these findings prompted us to investigate the localization and intra – cellular distribution of the neurotrophins and their relative receptors, to determine whether these growth factors participate in the transformation and development of the human pituitary adenomas. Although most pituitary microadenomas can be completely excised, it is difficult to totally remove invasive pituitary macroadenomas and, until now, no routine markers have been available to predict the aggressive behavior or recurrence of this lesion. However, Ki-67, a nuclear antigen recognized by the monoclonal antibody MIB-1 has proved to be useful in assessing several brain tumors, providing information about cell proliferation and, consequently, long – term prognosis (24 – 28). Generally, the Ki-67 labeling index (LI) in pituitary adenomas is relatively low in comparison to other

brain tumors (29 – 31), in which it is employed, together with a thorough clinical, hormonal and MR image follow – up, to identify the predicted recurrence of the tumor as early as possible. Hence, we decided to investigate, together with the analysis of the neurotrophins pattern, the expression level of MIB-1 (anti-Ki67) to define its possible role as a prognostic factor in pituitary adenomas.

Materials and Methods

Patients

Small specimens of human GH – secreting pituitary adenomas were harvested, during surgical operation, from 10 patients, and then processed for immunohistochemical analysis. Control specimens were obtained from 2 autaptic cases. Experiments were performed in compliance with the Italian laws and guidelines concerning the informed consent of patients (Dir. 2001/20/CE). The following molecules were investigated: nerve growth factor (NGF), brain - derived neurotrophic factor (BDNF), NT-3, NT-4, TrKA, TrKB, TrKC, p75NTR and MIB- 1.

Immunohistochemical analysis

For light microscope immunohistochemical analysis, small fragments of human GH – secreting adenomas were washed in PBS, fixed in 10% formalin and embedded in paraffin according to a standard procedure. Serial 4 µm thick sections were cut using a rotatory microtome, mounted on gelatine-coated slides and processed for immunohistochemistry. To study the immunolocalization of neurotrophins and their receptors, the antibodies we used were: i) rabbit anti-NGF polyclonal antibody (Santa Cruz Biotechnology, CA, USA), which display <1% cross-reactivity against recombinant human NT-3, NT-4 and BDNF; ii) rabbit anti-BDNF polyclonal antibody (Santa Cruz), which recognizes the amino-terminus of mouse BDNF and does not cross-react with NT-3 or NGF; iii) rabbit anti- NT3 polyclonal antibody (Santa Cruz

Biotechnology), raised against the amino-terminus of mouse NT-3, which does not cross-react with NGF or BDNF; iv) rabbit anti-NT4 polyclonal antibody (Santa Cruz Biotechnology); v) rabbit anti- TrKA polyclonal antibody (Santa Cruz Biotechnology), which recognizes an epitope corresponding to amino acids 763 to 777, mapping adjacent to the carboxy-terminus of human TrKA p140 and is not cross-reactive with TrKB or TrKC; vi) rabbit anti-TrKB polyclonal antibody (Santa Cruz Biotechnology), which recognizes an epitope corresponding to amino acids 794 to 808 of mouse TrKB p145 and is not cross-reactive with TrKA or TrKC; and vii) rabbit anti-TrKC polyclonal antibody (Santa Cruz Biotechnology), which recognizes an epitope corresponding to amino acids 798 to 812 of porcine TrKC p140 and is not cross-reactive with TrKA or TrKB; viii) goat polyclonal antibody to human NGF receptor p75 (Santa Cruz Biotechnology), which recognizes the amino acid sequence mapping the carboxy-terminus of the NGF receptor p75 precursor of human origin and is not cross-reactive with other growth factor receptors; ix) MIB-1, a rabbit polyclonal antibody raised against amino acids 2641-2940 mapping at the C-terminus of Ki-67 of human origin (Santa Cruz, CA, USA). Incubation with primary antibodies was performed overnight at 4°C at a final concentration of 2-5 µg/ml. Optimal antisera dilutions and incubation times were assessed in a series of preliminary experiments. After exposure to the primary antibodies, slides were rinsed twice in phosphate-buffer and incubated (1h and 30 min at room temperature) with the appropriate secondary antibody conjugated to horseradish peroxidase (HRP) (final dilution 1:100). The secondary antibody-HRP linked against rabbit immunoglobulins was purchased from Boehringer (Boehringer Mannheim GmbH, Mannheim, Germany), while secondary antibodies-HRP linked against mouse and goat immunoglobulins were from Sigma (Sigma Chemicals Co, St. Louis, MO, USA). After a further wash with phosphatebuffer, slides were treated with 0.05% 3,3-diaminobenzidine and 0.1% H₂O₂. Finally, sections were counterstained with Mayer's hematoxylin and observed by using a light microscope. To block endogenous peroxidase activity, slides were pre-treated with 3% H₂O₂, whereas the non-specific binding of immunoglobulins was prevented by adding 3% fetal calf serum to the

incubation medium. The intensity of the immune reaction was assessed microdensitometrically using an IAS 2000 image analyzer (Delta Sistemi, Rome, Italy) connected via a TV camera to the microscope. The system was calibrated taking as zero the background obtained in sections exposed to nonimmune serum. Ten 100 μm^2 areas were delineated in each section by a measuring diaphragm. Quantitative data regarding the intensity of the immune staining were analyzed statistically by analysis of variance (ANOVA) followed by Duncan's multiple range test as a *post hoc* test.

Results

Immunohistochemistry

Sections of GH – secreting adenomas samples exposed to the primary/secondary antibodies developed a dark-brown (intense), yellow-brown (slight) or no appreciable immunostaining. Immunoreactivity was specific since no immunostaining was obtained in positive control sections incubated with each primary antibody adsorbed with the specific peptide or with pre-immune serum (data not shown). Immunolabeling was located in the epithelial glands (described as epithelial neoplastic cells), extracellular matrix (ECM) and vessel endothelium (see the Table). Immunoreaction for NGF was moderate on epithelial glands (ep) and also appeared weak in the ECM (em), but was totally absent on the endothelium of the vessels (en) (Fig. 1A). Similarly TrkA immunoreactivity was moderate on epithelial glands (ep) and less appreciable in the ECM (em), but absent on the vessel endothelium (en) (Fig. 1B). A weak immunoreactivity for the p75NTR was described on epithelial glands (ep) and in the ECM (em), while it was absent on the endothelium of vessels (en) (Fig. 1C). BDNF immunoreactivity was highly appreciable in the ECM (em) and on epithelial glands (ep), but it resulted moderate on the vessel endothelium (en) (Fig. 2A). A strong immunoreactivity for TrkB was observed in the ECM (em), appreciable in the vessel endothelium (en) and weak on epithelial glands (ep) of the neoplastic area (Fig. 2B). A very strong immunoreactivity was revealed by NT-3 and TrkC in the ECM (em), an appreciable expression also on the

vessel endothelium (en) and a weak immunoreactivity on epithelial glands (ep) for these factors (Fig. 3A - 3B). Finally MIB – 1 immunoreactivity was weakly expressed on epithelial glands (ep) and on the vessel endothelium (en) (Fig. 4A - 4B). Illustrations regarding autaptic findings (2 cases) are not provided because they were not significant.

Discussion

Pituitary adenomas account for 10-15% of all primary brain tumors and benign tumors and are usually slow growing (1 – 4). In particular, growth hormone-secreting adenomas account for about 20 % of all pituitary adenomas. GH-producing adenomas have been histology classified into densely and sparsely granulated adenomas (32). They derive from the acidophil cell line. This lineage, comprising somatotroph, mammotroph and thyrotroph cells, is controlled by a common transcription factor named PIT-1. Previous studies suggest that neurotrophins, especially NGF, may be involved in pituitary endocrine cell proliferation, growth and differentiation (13 – 14). NGF is a particularly interesting neurotrophin in pituitary function and physiology (15 – 18). Immunohistochemical studies have also shown that NGF is selectively expressed by somatotroph, mammotroph and thyrotroph cells, stored in secretory granules. Moreover, neurotrophins and their receptors have been shown to play a role in cancer, promoting tumor progression. In particular, NGF cooperates with EGF and bFGF in directing differentiation of lactotrophs cells stimulating PRL synthesis and D-2 dopaminergic receptor expression (21), via binding to p75NTR and activation of NF – kB in a TrkA – independent way. In fact, short – term treatment of patients with bromocriptine – resistant prolactinomas, with NGF resulted in differentiation of the tumors into less aggressive, lactotrope – like cells, re – expressing D-2 receptors, thus restoring the molecular target for conventional therapy with D-2 agonists (21). TrkA as well as TrkB expression has been found in tumor and non – tumoral adenohypophyseal cells from human pituitary adenomas by Aguado et al (14). However the localization of the neurotrophin receptors in human normal

pituitary gland and their expression in human pituitary adenomas is still unclear. In our observations we described a moderate immunoreaction for NGF, and its relative receptor TrKA and p75NTR, on epithelial glands and less appreciable in the ECM, similarly to the experimental observations of Assimakopoulou et al (13) but different to the Aguado's et al findings (14), thus suggesting the role of NGF and its signaling in the development of normal pituitary gland but not in the progression and proliferation of the pituitary adenomas. BDNF and its receptor TrKB resulted highly appreciable in the ECM and moderate on the vessel endothelium, suggesting their role in the proliferation of a neoplastic microenvironment, involving the extracellular matrix components and the angiogenetic network, given that vascular endothelial cells synthesize and secrete BDNF (32). On the other hand a very strong immunoreactivity was revealed by NT-3 and TrKC in the ECM, and an appreciable expression also on the vessel endothelium, differently from the Aguado's et al findings (14), leading us to describe this neurotrophin as the principal neurotrophic factor involved in the progression of pituitary adenomas. This discrepancy may be due to different immunohistochemical experimental protocols. Considering the efficiency of the antibodies used for this investigation, the expression revealed by these molecules in human pituitary adenomas (GH – secreting) suggests a functional direct role of BDNF and NT-3 on this tumors. Finally MIB – 1 resulted weakly detectable in epithelial glands, but totally absent on the vessel endothelium, confirming the low proliferation rate of Gh-producing adenomas which generally show a label index less than 3 %. However the distribution and localization of all these factors in human pituitary adenomas (GH – secreting) still remains unclear. Additional studies would be necessary to better explain the biological role of these molecules in the development and progression of this type of tumors.

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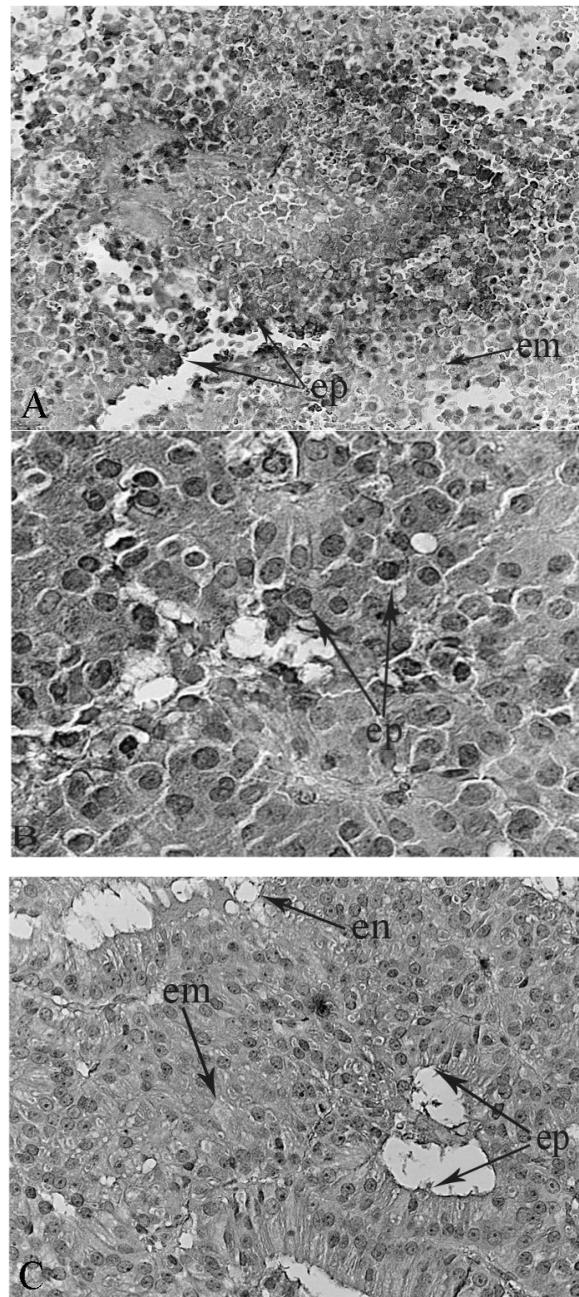


Fig. 1. Micrographs of NGF-TrKA-P75 immunostaining in GH-secreting pituitary adenomas. NGF immunoreactivity was moderate in the epithelial compartment of the neoplastic area, weak in the extracellular matrix (em) and totally absent in the vessel endothelium (en) (not shown)(**A**: 20X). Immunoreaction for TrKA was moderate in the nucleus of the neoplastic cells, weak in the extracellular matrix (not shown) and totally absent in the vessel endothelium (en) (not shown) (**B**: 40X). P75NTR immunoreactivity was weak in the epithelial neoplastic cells and in the extracellular matrix (em), but totally absent in the vessel endothelium (en) (**C**: 20X).

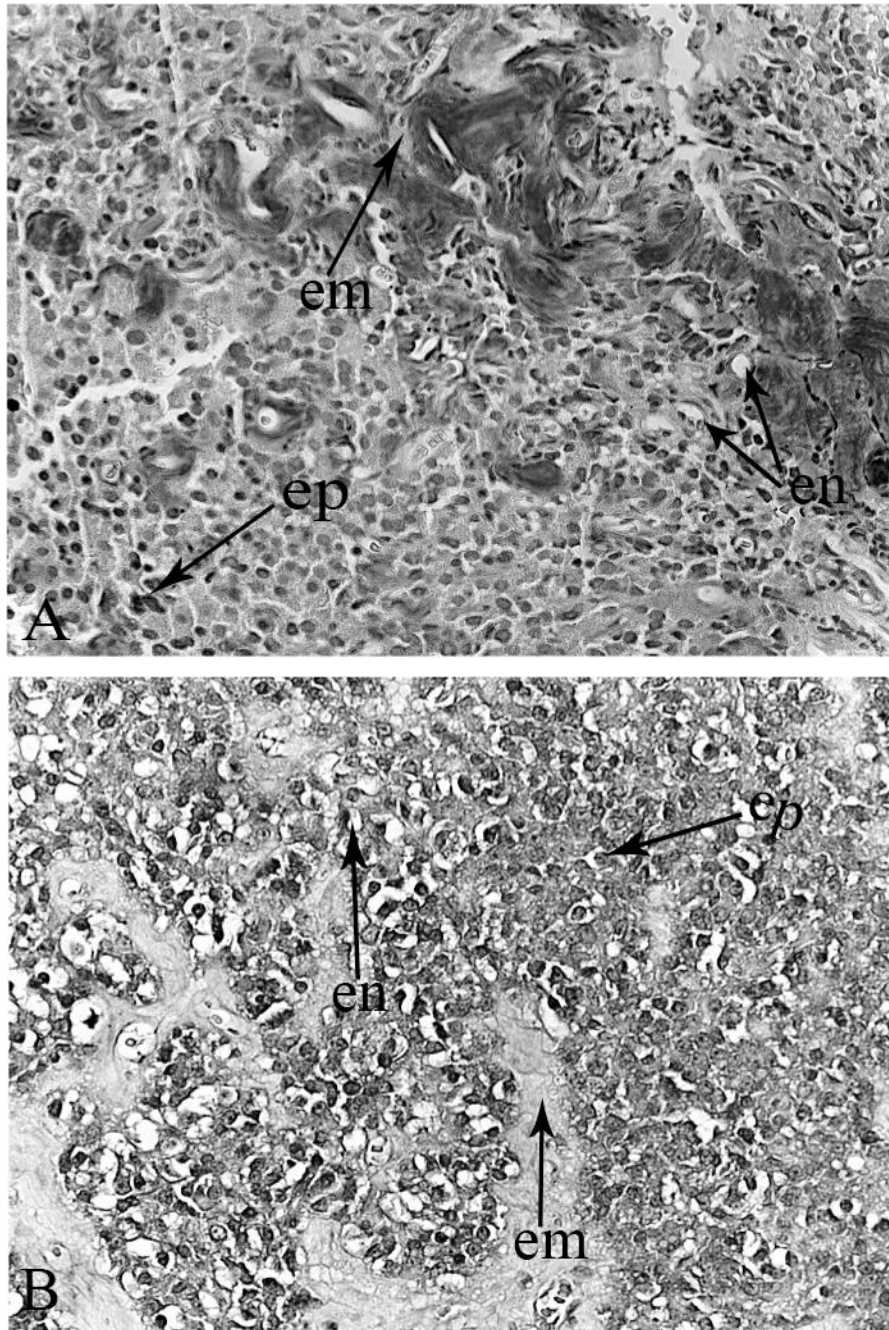


Fig. 2. Micrographs of BDNF-TrkB immunostaining in GH-secreting pituitary adenomas. BDNF showed a significantly high expression in the extracellular matrix (em) and on epithelial glands, but moderate on the vessel endothelium (en) (**A**: 20X). TrkB Immunoreactivity was strong in the extracellular matrix (em), appreciable in the vessel endothelium (en) and weak on epithelial glands of the neoplastic area (**B**: 20X).

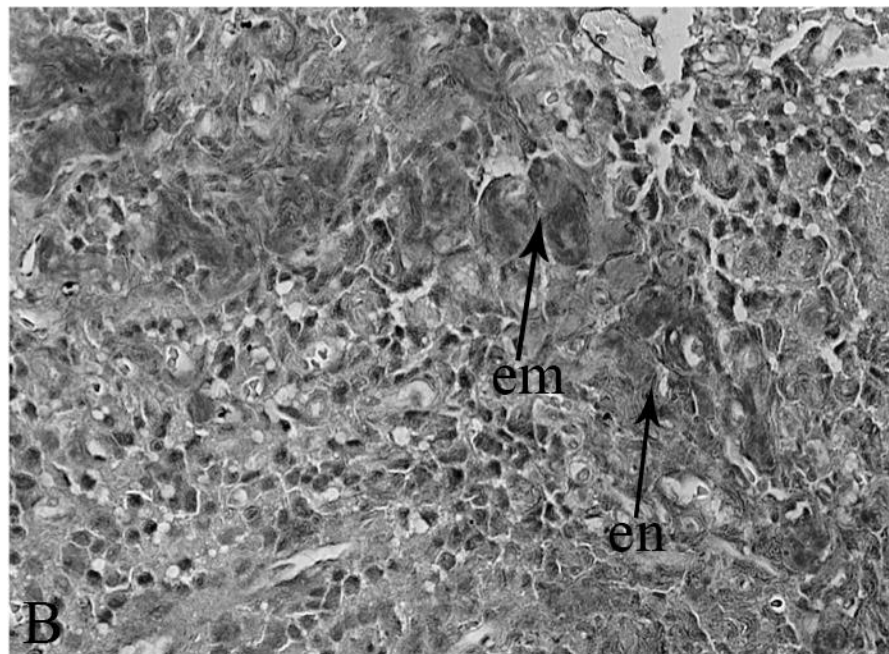
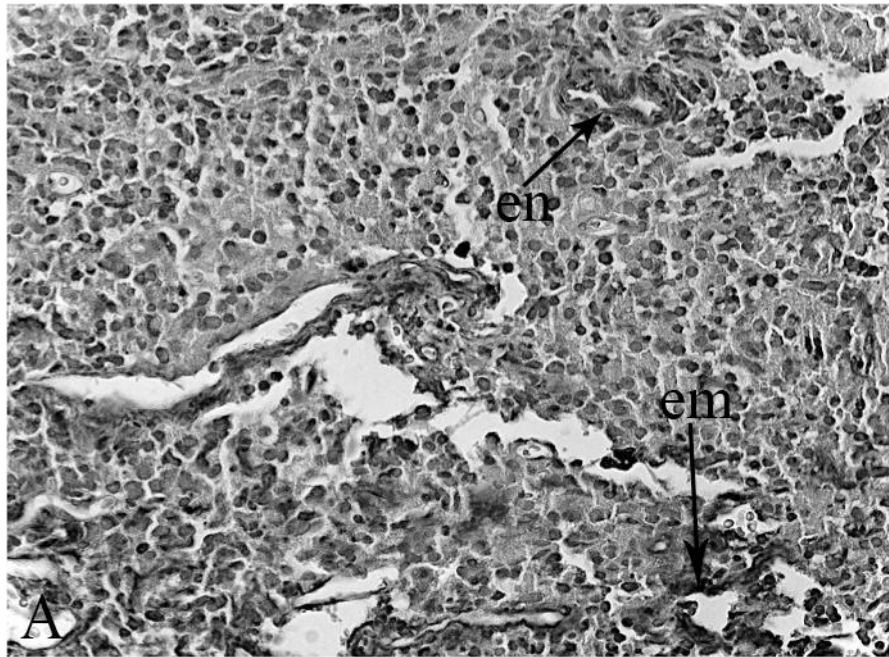


Fig. 3. Micrographs of NT3-TrKC immunostaining in GH-secreting pituitary adenomas. NT-3 was strongly expressed in the extracellular matrix (em) and on the vessel endothelium (en), but it was moderate expressed in the epithelial neoplastic cells. (**A:** 20X). A strong immunoreactivity for TrKC was observed in the extracellular matrix (em) and a weak expression on the vessel endothelium (en) and on epithelial glands (ep) (**B:** 20X).

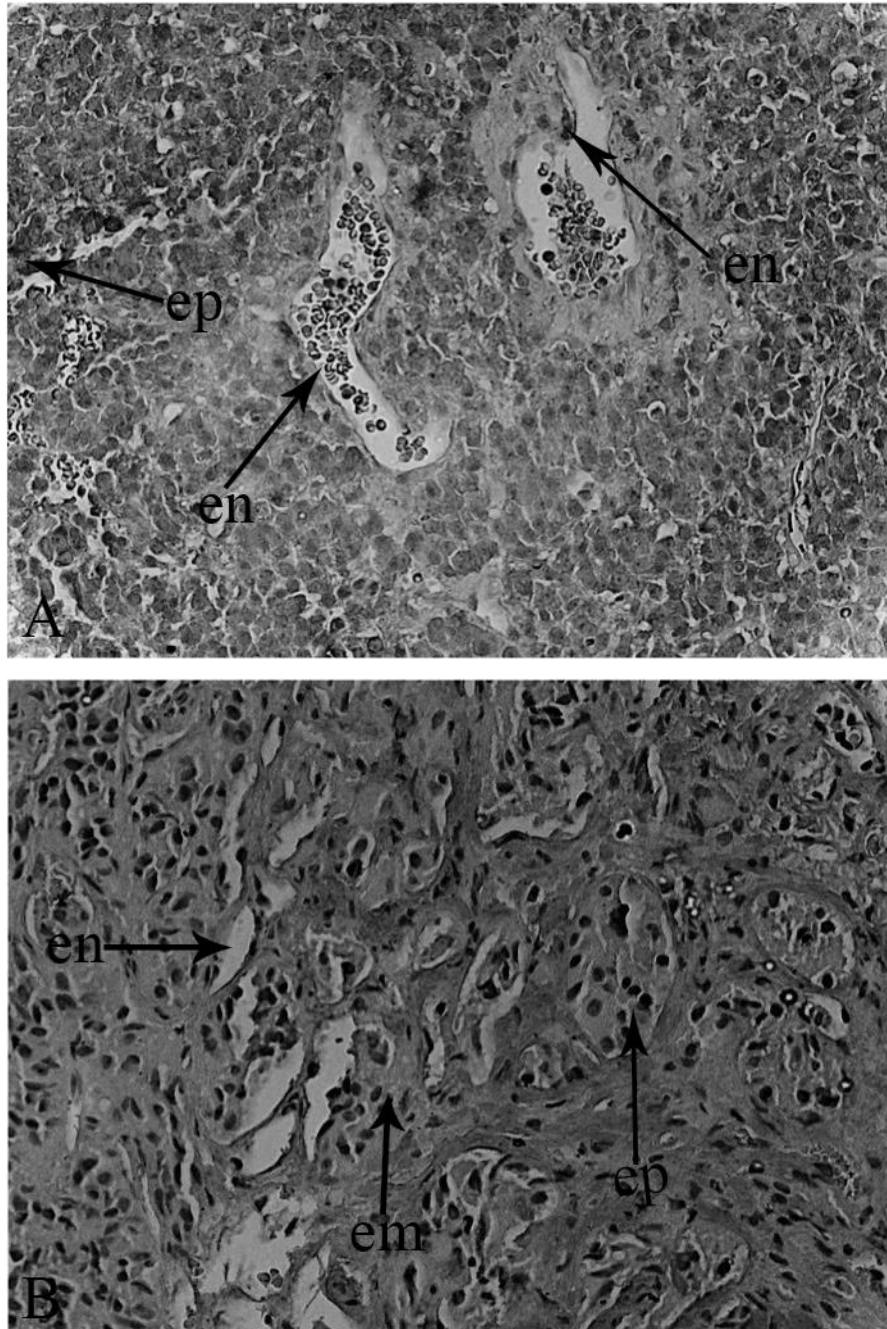


Fig. 4. Micrographs of Ki-67 immunostaining in GH-secreting pituitary adenomas. Ki-67 immunoreactivity was weak on epithelial neoplastic cells and on the vessel endothelium (en), but it was relevant in the extracellular matrix (em) (**A, B**: 20X).

Table I. Results of the immunohistochemical analysis for NTs and NT receptors in human GH-secreting pituitary adenomas.

	Epithelial glands	Extracellular matrix	Vessel endothelium
NGF	+	+/-	-
BDNF	++	+++	+
NT3	+	+++	++
MIB-1	+/-	++	-
TrkA	+	+/-	-
TrkB	+/-	++	+
TrkC	+/-	+++	++
P75NTR	+/-	+/-	-

+++ : strong immunoreactivity; ++ : relevant immunoreactivity; + : moderate immunoreactivity; +/- : weak immunoreactivity; - : absence of immunoreactivity

Table II. Results of the immunohistochemical analysis in percentages.

	Epithelial glands	Extracellular matrix	Vessel endothelium
NGF	±50%	±25%	±0%
BDNF	±75%	±100%	±50%
NT3	±50%	±100%	±75%
MIB-1	±25%	±75%	±0%
TrkA	±50%	±25%	±0%
TrkB	±25%	±75%	±50%
TrkC	±25%	±100%	±75%
P75NTR	±25%	±25%	±0%

+++ : strong immunoreactivity; ++ : relevant immunoreactivity; + : moderate immunoreactivity; +/- : weak immunoreactivity; - : absence of immunoreactivity